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TITLE: Functional Validation of H2 Relaxin and Its Downstream Effectors as Mediators, Therapeutic Targets, and Potential Biomarkers of Prostate Cancer Progression

PRINCIPAL INVESTIGATOR: Ralph W. deVere White, M.D.

CONTRACTING ORGANIZATION: University of California, Davis
Davis, CA 95616

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14. ABSTRACT Currently, castrate resistant prostate cancer (CRPC) remains incurable. The identification of novel pathways that promote castrate resistant growth of prostate cancer (CaP) cells is critical for the development of successful new therapies to treat CaP. Our group has identified H2 relaxin as a facilitator of CRPC. In the past year, we have generated data that supports a simultaneous inhibition of PKA and B-catenin (pathways activated by H2 relaxin) causes increased inhibition of both castrate resistant growth and AR signaling, H2 relaxin promotes neuroendocrine differentiation through activation of PKA, steroid hormones cause transactivation of the H2 relaxin promoter, H2 relaxin causes decreased expression of IkappaB-alpha, increased expression of Bcl-2 and increased translocation of NFkappaB to the nucleus, LNCaP stably transfected with H2 relaxin are more resistant to treatment with chemotherapeutic agents, and that inhibition of IKK decreases H2 relaxin mediated chemo-resistance to perifosine. This data implies that H2 relaxin-mediated activation of the PKA and NFkappaB signaling pathways promotes CR growth and chemo-resistance respectively.					
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Introduction

Currently, castrate resistant prostate cancer (CRPC) remains incurable. The identification of novel pathways that promote castrate resistant growth of prostate cancer (CaP) cells is critical for the development of successful new therapies to treat CaP. Our group has identified H2 relaxin as a facilitator of CRPC (1). We have demonstrated that blocking H2 relaxin expression, or expression of LGR7, the H2 relaxin receptor, can inhibit castrate resistant growth, and that the H2 relaxin signaling pathway in part mediates CRPC by promoting interaction between β -catenin and the androgen receptor (AR) (2). Our hypothesis is that H2 relaxin, and its downstream effectors, represent therapeutic targets for use in the prevention and treatment of CRPC, and that H2 relaxin itself can potentially be used as a biomarker to predict CaP progression in a subset of CaP patients. To test this hypothesis we proposed the following specific aims: (1) To further elucidate the mechanism(s) by which the H2 relaxin mediates CRPC, (2) To functionally validate H2 relaxin-mediated CaP progression to CRPC using *in vivo* models of CaP, and to use *in vivo* models of CRPC to determine whether blocking expression H2 relaxin is a viable treatment option for CRPC, (3) To establish the usefulness of H2 relaxin as a biomarker for progression to CRPC.

Body:

Task 1 - *To further delineate the pathways(s) by which H2 relaxin mediates AI CaP.* Our preliminary data indicated that H2 relaxin can activate the cAMP/PKA pathway in LNCaP cells (data not shown). Our previous studies demonstrated that H2 relaxin can mediate CRPC by causing PI3K-dependent B-catenin stabilization, co-localization of B-catenin and AR, co-translocation of the B-catenin/AR complex into the nucleus and transactivation of the PSA promoter (2, 6). However, inhibition of B-catenin using specific siRNA caused only partial decrease in H2 relaxin-mediated CR growth and AR activation. To determine whether simultaneous inhibition of PKA and B-catenin caused further inhibition of H2 relaxin-mediated CR growth and AR activation, we transfected LNCaP-rlx clones with B-catenin-specific siRNA and then treated them with H89. Approximately 50% B-catenin inhibition was achieved 24 hours post-transfection and maintained until 5 days post-transfection (Fig. 4.A). The LNCaP-rlx clones were treated with H89 24 hours post-transfection. Castrate resistant growth and PSA levels were measured 3 days post-treatment with H89. The simultaneous inhibition of PKA and B-catenin caused a further decrease in CR growth and PSA levels compared to transfection of B-catenin siRNA or treatment with H89 alone (Fig. 4.B and 4.C). These data demonstrate that both pathways play a role in facilitating H2 relaxin-mediated CR growth.

H2 relaxin promotes neuroendocrine differentiation through activation of PKA. As increased intracellular cAMP levels have been shown to induce neuroendocrine (NE) characteristics in prostate cells (49) we decided to also look at the effect of H2 relaxin on markers of NE differentiation (NED). Addition of rh H2 relaxin to LNCaP or stable transfection of LNCaP with H2 relaxin caused a significant increase in mRNA and protein levels of neuron specific enolase (NSE), a well established marker of NED. Conversely, increased expression of H2 relaxin caused a significant decrease in expression of neutral endopeptidase (NEP), an enzyme that is expressed at high levels by normal CaP cells and which is responsible for breaking down neuropeptides such as bombesin (also known as GRP), ET-1 and neurotensin (50, 51) that have been shown to promote AI CaP (52). The combined data indicate that H2 relaxin may play a role in promoting NED. To determine whether expression of NSE and NEP was controlled by H2 relaxin-mediated activation of PKA, we then inhibited PKA activation in LNCaP stably transfected with H2 relaxin (LNCaP-rlx) using H89. Inhibition of PKA resulted in a dramatic decrease in NSE mRNA expression in both LNCaP-rlx sublines, there was a lesser effect on NEP levels. Inhibition of PKA did not appear to alter NSE or NEP protein levels however, indicating that NSE protein may be stabilized by other factors.

Steroid hormones cause transactivation of the H2 relaxin promoter. To determine which factors affect H2 relaxin we employed a fusion plasmid that expresses luciferase under the control of the H2 relaxin promoter (kind gift of Dr. Bryant-Greenwood). Dr. Bryant-Greenwood has previously demonstrated that addition of progesterone or glucocorticoid to a placental choriocarcinoma cell line (JAR) caused increased transcription of H2 relaxin. As several steroid hormones are used to treat metastatic CaP, we were interested in determining

whether such treatments could also cause increased expression of H2 relaxin in CaP cells and thereby potentiate disease progression. Luciferase assays determined that all of the steroid hormones tested (hydrocorticoid, prednisone, R1881, DHEA, dexamethasone) caused elevated transactivation of the H2 relaxin promoter. IL-6, a cytokine associated with NED, also caused increased transactivation of H2 relaxin. This finding confirms previous findings that demonstrated that treatment of LNCaP with IL-6 and epinephrine leads to upregulation of H2 relaxin expression (15).

H2 relaxin causes decreased expression of IkappaB-alpha, increased expression of Bcl-2 and increased translocation of NFkappaB to the nucleus. Although not specified in the statement of work, through interaction with Dr. Allen Gao's lab we have also started to elucidate the interaction between H2 relaxin and the NFkappaB pathway. Expression of NFkappaB represents an independent risk factor for recurrence in CaP patients following prostatectomy (25, 26). In addition, increased activity of NFkappaB and expression of Bcl-xL have been linked to increased chemo-resistance in several cancer types, including breast cancer (27). Several studies have demonstrated that the NFkappaB signaling pathway is active in CRPC. To determine whether the NFkappaB pathway is active in the LNCaP-rlx clones we assessed IkappaB-alpha expression levels and phosphorylation state, and NFkappaB localization. IkappaB-alpha levels were significantly lower in the LNCaP-rlx clones and was shown to be phosphorylated indicating active degradation of IkappaB occurs in these cells (Fig. 5.A). Increased expression of Bcl-xL, a downstream effector of NFkappaB, was also observed. Increased expression of Bcl-xL has been associated with increased chemo-resistance in CaP cells. Immunocytochemical analysis of the LNCaP-rlx clones revealed a significant increase in levels of nuclear NFkappaB compared to LNCaP clones. LNCaP treated with TNF-alpha was used as a positive control.

LNCaP stably transfected with H2 relaxin are more resistant to treatment with chemotherapeutic agents.

We had previously noted that the LNCaP-R273H subline is more chemo-resistant compared to LNCaP-vector. As the R273H p53 mutant is able to bind to the H2 relaxin promoter and causes its transactivation we asked whether H2 relaxin played a role in mediating this chemo-resistant phenotype. Annexin V/propidium iodide (PI) labeling followed by flow cytometry analysis confirmed that the LNCaP-rlx#3 and -rlx#5 sublines are more resistant to treatment with LY294002 (PI3K inhibitor), perifosine (Akt inhibitor), rapamycin (mTOR inhibitor) and docetaxel (anti-mitotic) (Figure 2A and B). In LNCaP-vector, treatment with vehicle control, LY294002, perifosine, rapamycin and docetaxel induced ~10, 35, 14, 36 and 26% early apoptosis respectively (Fig. 1A). In LNCaP-rlx#3, these treatments induced ~4, 18, 12, 13 and 8% early apoptosis. In LNCaP-rlx#5, these treatments induced ~1, 3.5, 7, 2.5, 1.5% early apoptosis. Early apoptotic cells are characterized as being Annexin V positive and PI negative. Expression of annexin V indicates the presence of externalized phosphatidyl serine (PS) and that programmed cell death is actively occurring. Lack of PI staining indicates that the cell membrane is still intact and therefore that cell death has not yet occurred. All of the four drug treatments caused less early apoptosis in the LNCaP-rlx clones, however, treatment with docetaxel appeared to have the least effect on levels of early apoptosis. A similar trend was observed when the percentage of cells in late apoptosis was measured in response to treatment with vehicle control, LY294002, perifosine, rapamycin or docetaxel (Fig. 2.B). In LNCaP-vector, these treatments induced ~1.1, 2.8, 8.8, 1.2 and 2.8% late apoptosis. In LNCaP-rlx#3 ~0.4, 0.8, 0.8, 1.1 and 0.6% late apoptosis was observed and in LNCaP-rlx#5 ~0.2, 0.2, 2.4, 0.2 and 0.2% late apoptosis. Of note is the fact that treatment with perifosine induced relatively little early apoptosis but did induce a significantly larger late apoptosis response.

Inhibition of IKK decreases H2 relaxin mediated chemo-resistance to perifosine.

IKK causes phosphorylation of IkappaB-alpha and subsequent proteasome-mediated degradation. This degradation allows NFkappaB to translocate to the nucleus. Inhibition of IKK caused a small but significant increase in perifosine-mediated early and late apoptosis in the LNCaP-rlx clones, indicating that the NFkappaB signaling pathway can mediate chemo-resistance in the LNCaP-rlx clones.

Task 2 – To functionally validate H2 relaxin-mediated AI CaP growth using in vivo models of CaP, and to use in vivo models of AI CaP to determine whether blocking expression H2 relaxin and/or its downstream effectors is

a viable treatment option for AI CaP. Task 2 studies are expected to be initiated in the upcoming year of this grant and are not reported in this annual report.

Task 3 - *To establish the usefulness of H2 relaxin as a biomarker for progression to AI CaP.* Task 3 studies are expected to be initiated in year 3 of the grant and are not included in this annual report.

Key research accomplishments

Research accomplishments associated with Task 1 in the statement of work; Delineation of the pathway(s) by which H2 relaxin mediates progression to castrate resistant prostate cancer.

- Simultaneous inhibition of PKA and B-catenin (pathways activated by H2 relaxin) causes increased inhibition of both castrate resistant growth and AR signaling
- H2 relaxin promotes neuroendocrine differentiation through activation of PKA.
- Steroid hormones cause transactivation of the H2 relaxin promoter.
- H2 relaxin causes decreased expression of IkappaB-alpha, increased expression of Bcl-2 and increased translocation of NFkappaB to the nucleus.
- LNCaP stably transfected with H2 relaxin are more resistant to treatment with chemotherapeutic agents.
- Inhibition of IKK decreases H2 relaxin mediated chemo-resistance to perifosine.

Reportable outcomes

Published abstracts:

1. Ruth Vinall, Ibitola Asaolu, Xu-Bao Shi and Ralph deVere White. H2 relaxin facilitates castrate resistant growth of prostate cancer cells by a mechanism that involves nuclear translocation of NFkappaB. American Association for Cancer Research Special Conference; Advances in Prostate Cancer Research. 2009 Jan 21-24th; San Diego, CA.
2. Ruth Vinall, Shangqin Liu, Hsing-Jien Kung and Ralph deVere White. H2 relaxin can influence the expression of molecules associated with neuroendocrine differentiation. Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA.

Generation of cell lines'

1. LNCaP that stably express H2 relaxin (LNCaP-rlx#3 and LNCaP-rlx#5).

Conclusions

As part of this study we have generated and characterized 2 LNCaP sublines that overexpress H2 relaxin; LNCaP-rlx#3 and -rlx#5. These LNCaP-rlx sublines are able to grow in CR conditions and express high levels of PSA, confirming our previous finding that treatment of LNCaP with recombinant H2 relaxin can mediate CR growth and activation of the AR pathway. In the current study we have used these LNCaP-rlx sublines to further elucidate the mechanisms by which H2 relaxin mediates CR growth. Our data imply that H2 relaxin-mediated activation of the PKA and NFkappaB signaling pathways promotes CR growth and chemo-resistance respectively.

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ABSTRACTS – 2008-2009

American Association for Cancer Research Special Conference; Advances in Prostate Cancer Research. 2009 Jan 21-24th; San Diego, CA.

H2 relaxin facilitates castrate resistant growth of prostate cancer cells by a mechanism that involves nuclear translocation of NFkappaB.

Ruth L. Vinall, Ibitola Asaolu, Xu-Bao Shi and Ralph deVere White.

Currently, castrate resistant (CR) prostate cancer (PC) remains incurable. The identification of novel pathways that promote androgen independent growth of prostate cancer cells is critical for the development of successful new therapies to treat CaP. Our group has identified H2 relaxin as a facilitator of castrate resistant prostate cancer (CRPC) (Vinall et al., 2006). The mechanism by which H2 relaxin causes CRPC remains to be fully elucidated. We have demonstrated that H2 relaxin promotes interaction between β -catenin and the androgen receptor (AR) (Liu et al., 2007). However, blocking this pathway causes only a modest decrease in H2 relaxin-mediated CR growth. In the current study we have determined that H2 relaxin can also mediate CR growth by causing translocation of NFkappaB to the nucleus. As part of these studies we generated LNCaP sublines that stably overexpress H2 relaxin. As expected, these LNCaP-relaxin sublines are able to grow in the absence of androgen and express high levels of PSA. In addition we found that the LNCaP-relaxin sublines are more resistant to treatment with either docetaxel or perifosine compared to the LNCaP-vector control sublines. Immunocytochemistry was used to demonstrate that the LNCaP-relaxin sublines express higher levels of nuclear NFkappaB compared to LNCaP-vector only control sublines. Western blot analysis demonstrated that levels of IkappaB-alpha are decreased in the LNCaP-relaxin sublines while levels of Bcl-XL are increased. Inhibition of LGR7, the H2 relaxin receptor, using an LGR7-specific siRNA caused increased expression of IkappaB-alpha indicating that H2 relaxin signaling plays a role in promoting nuclear translocation of NFkappaB. The combined data indicate that nuclear translocation of NFkappaB is another mechanism by which H2 relaxin facilitates CRPC.

Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA.

H2 relaxin can influence the expression of molecules associated with neuroendocrine differentiation.

Ruth Vinall, Shangqin Liu, Hsing-Jien Kung and Ralph deVere White.

It has been shown that neuroendocrine differentiation (NED) is present, at least focally, in all cases of prostate cancer (CaP). NE cells secrete neuropeptides such as bombesin and neurotensin that cause the inappropriate proliferation of surrounding cells. Abundant literature has demonstrated a link between NED in CaP and progression towards an androgen-independent state. Our group previously identified H2 relaxin as a facilitator of androgen independent prostate cancer (AI CaP) (Vinall et al., 2006). Our current data suggest that H2 relaxin may facilitate AI CaP by promoting NED. RT-PCR and Western blot analysis demonstrated that stable transfection of LNCaP with H2 relaxin or addition of H2 relaxin to LNCaP cultured in charcoal stripped serum (CSS) resulted in up-regulation of neuron specific enolase (NSE) and chromogranin A expression and down-regulation of neutral endopeptidase (NEP). NSE and chromogranin A are considered markers of NE cells. NEP is an enzyme that is able to degrade neuropeptides such as bombesin and neurotensin. Further investigation using enzyme assays revealed that H2 relaxin can cause activation of cAMP (peak activity 10 minutes post-treatment) and PKA (peak activity 15 minutes post-treatment) and that inhibition of PKA using H-89 can prevent an H2 relaxin-mediated decrease in NEP expression. In conclusion, these data indicate that H2 relaxin may promote AI CaP by a mechanism that involves components associated with NED.

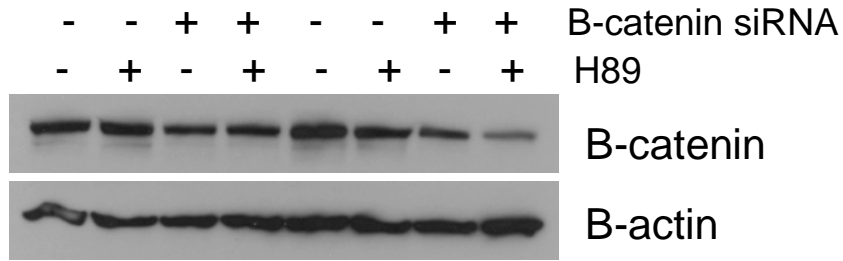
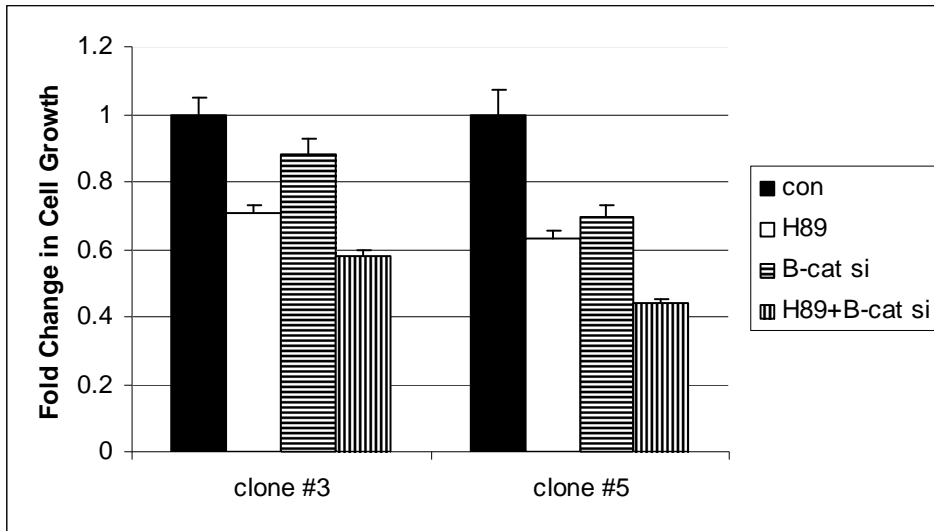
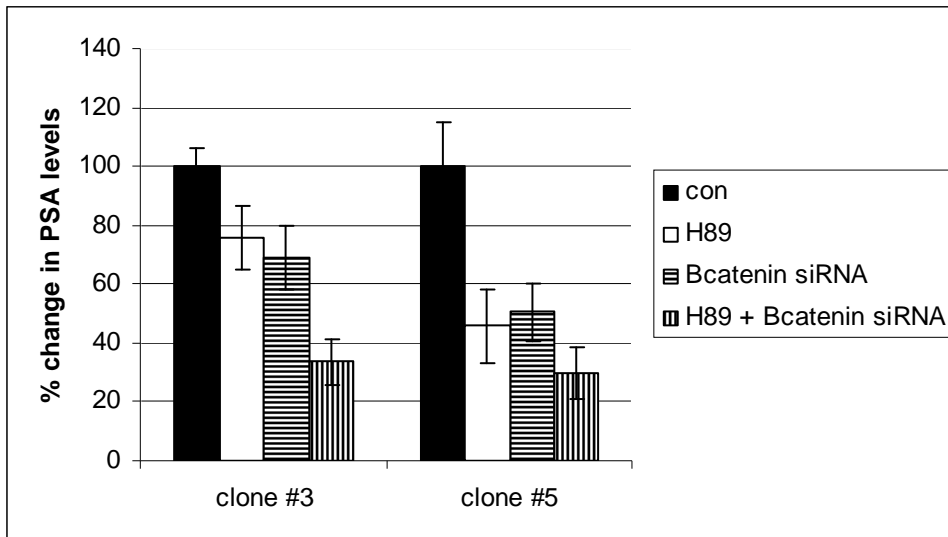
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Figure 1: Simultaneous inhibition of PKA and B-catenin (A) causes increased inhibition of cell growth (B) and expression of PSA (C) in LNCaP-relaxin sublines.

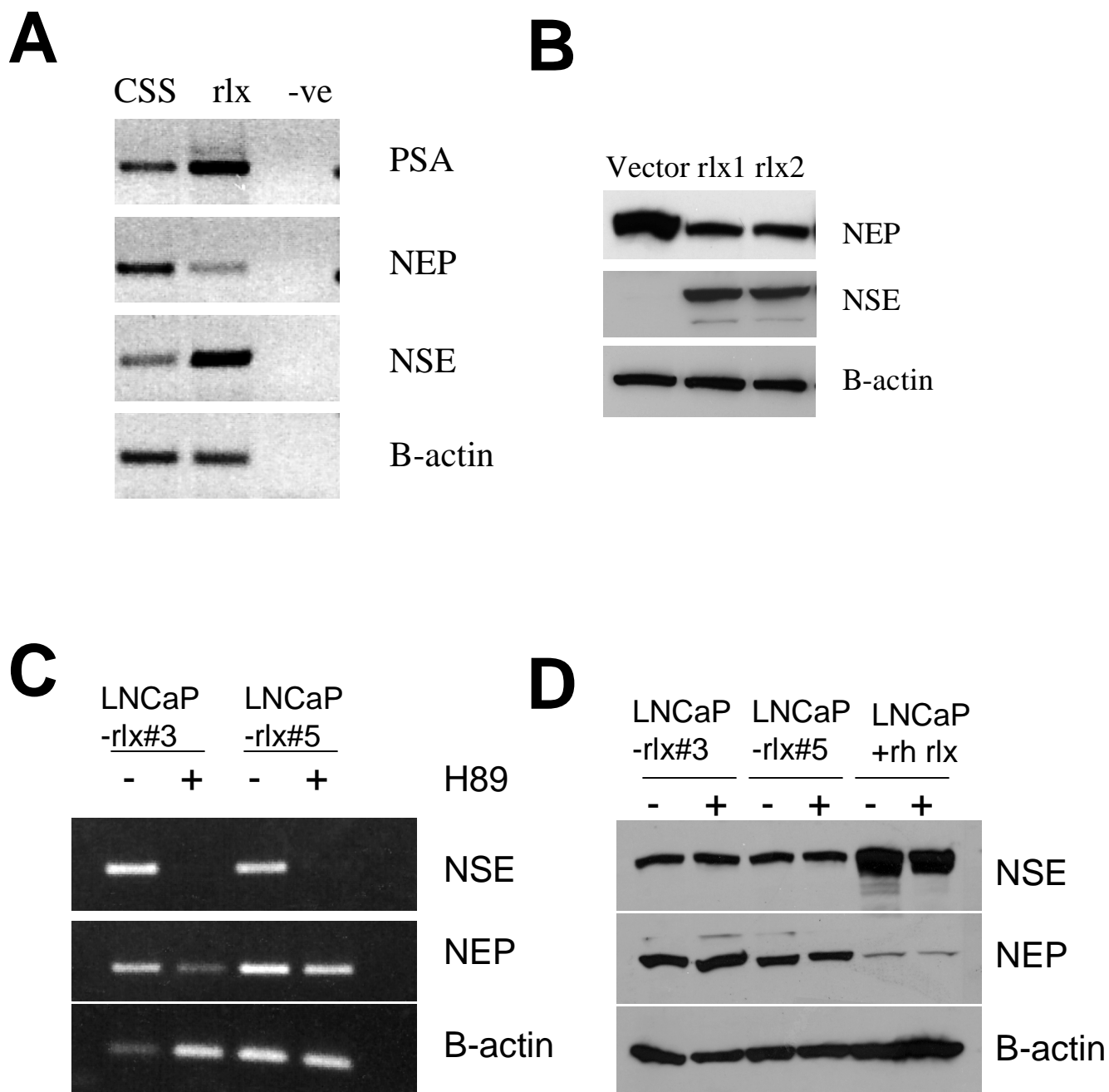


Figure 2: Overexpression of H2 relaxin results in decreased expression of NEP and increased expression of NSE at both the mRNA and protein level (A and B). Treatment of the LNCaP-relaxin sublines with H89, an inhibitor of PKA, caused a dramatic reduction in NSE mRNA expression (C) but no decrease in NSE Protein expression (D).

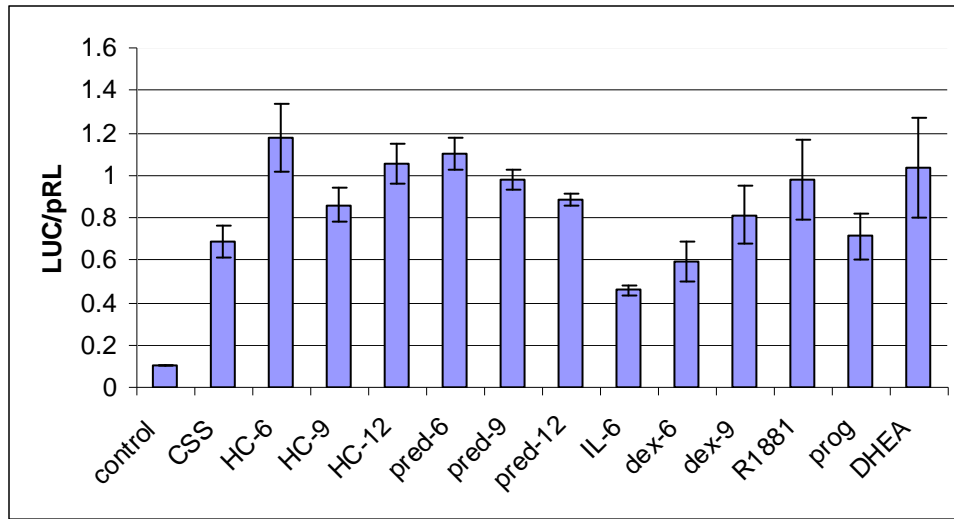


Figure 3: Parental LNCaP were transfected with a fusion plasmid containing the H2 relaxin promoter and the luciferase reporter gene. Addition of several steroid hormones, including hydrocorticoid (HC), prednisone (pred), dexamethosone (dex), synthetic androgen (R1881), progesterone (prog) and DHEA, resulted In increased luciferase expression demonstrating that these steroid hormones Are able to cause transactivation of the H2 relaxin promoter.

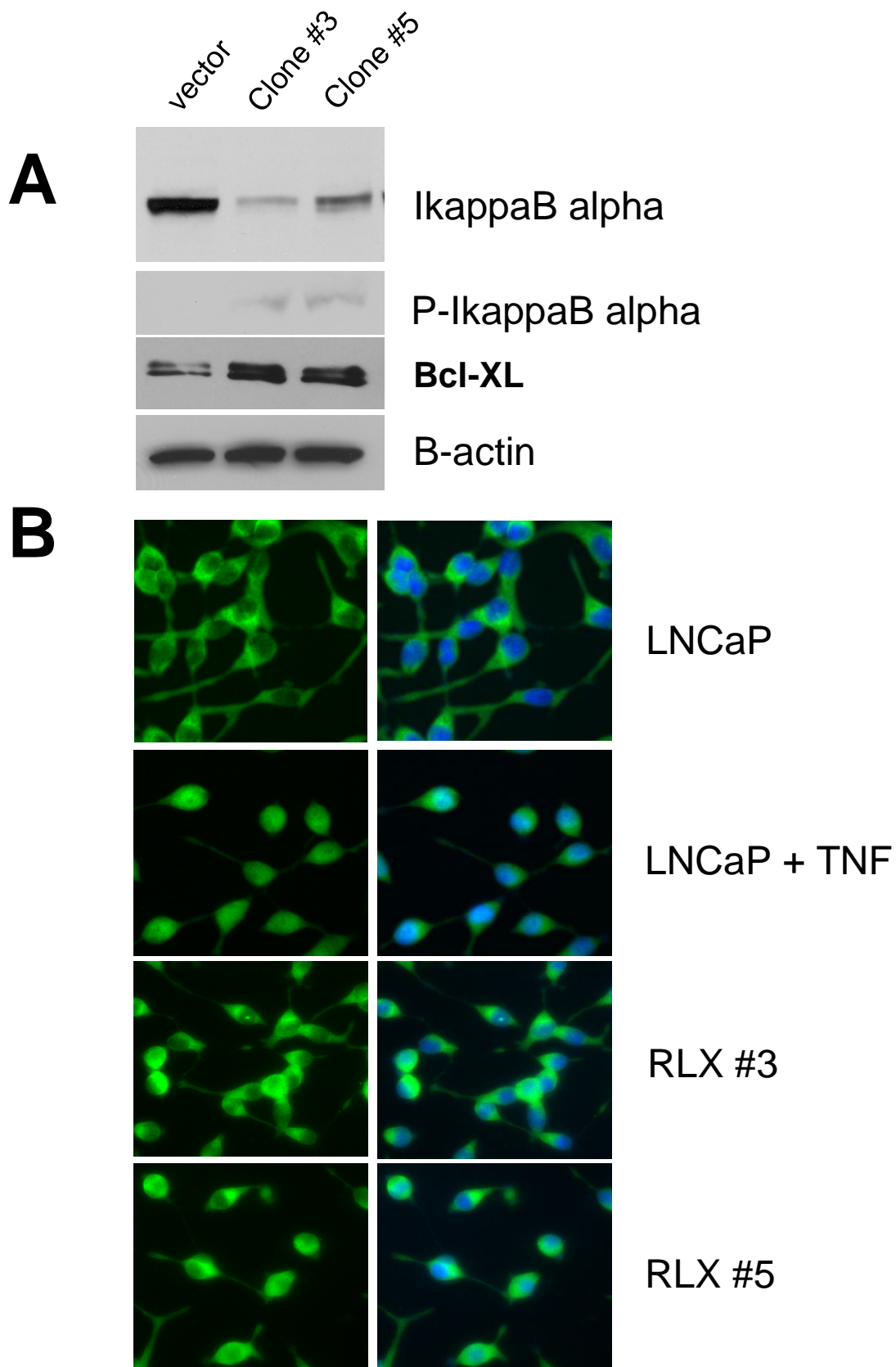


Figure 4: LNCaP-relaxin sublines express decreased levels of IkB alpha and increased levels of Bcl-XL (A). Increased nuclear translocation of NFkB is observed in LNCaP-relaxin sublines (B).

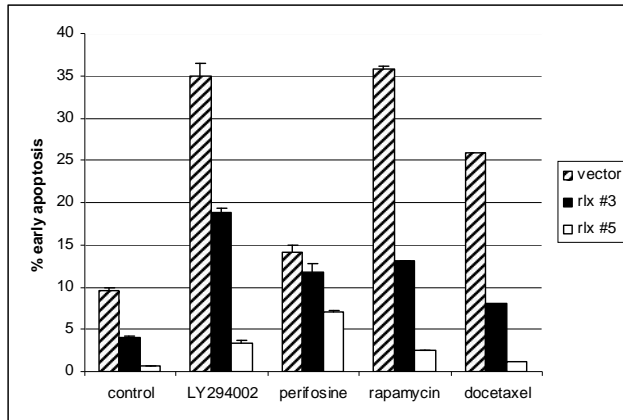
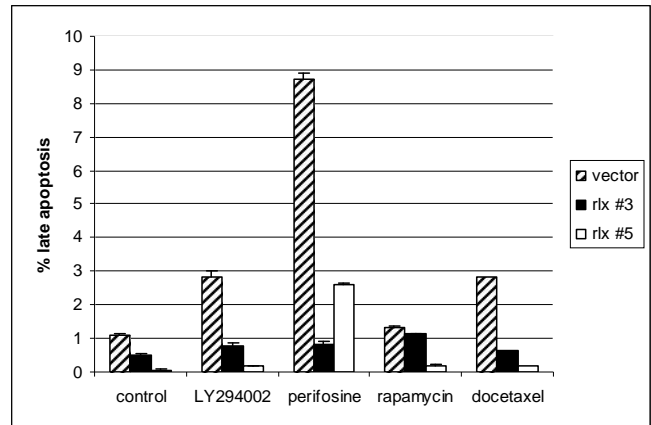
A**B**

Figure 5: LNCaP stably transfected with H2 relaxin (rlx #3, rlx #5) are more chemo-resistant to treatment with LY294002, perifosine, rapamycin and docetaxel than LNCaP stably transfected with vector alone. Both less early (annexin V only, A) and less late apoptosis (annexin V and propidium iodide, B) Was observed in the LNCaP-rlx sublines.

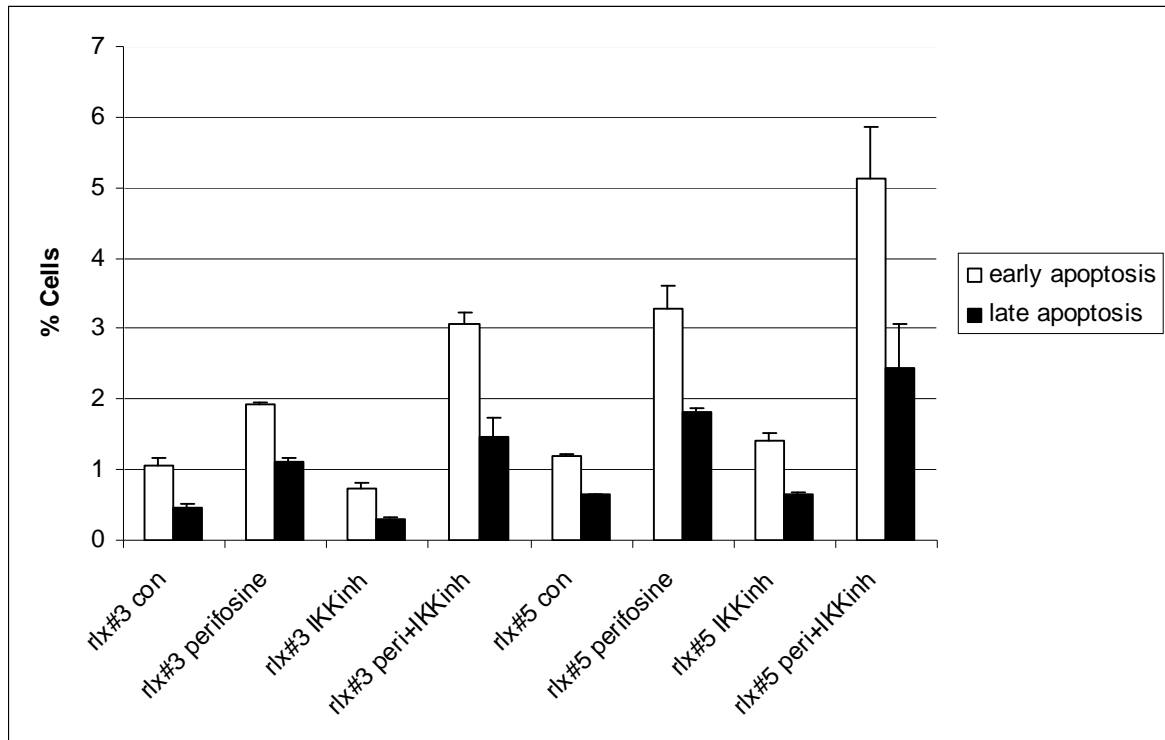


Figure 6: Inhibition of NFkB signaling pathway reduces chemo-resistance of LNCaP-relaxin clones.